FEBS Letters 443 (1999) 246–250 FEBS 21475

Cloning of the peroxiredoxin gene family in rats and characterization of the fourth member

Akio Matsumoto[a,b], Ayako Okado[a], Tsuneko Fujii[a], Junichi Fujii[a], Masayuki Egashira[c,d], Norio Niikawa[c], Naoyuki Taniguchi[a,*]

[a]*Department of Biochemistry, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan*
[b]*Department of Cardiovascular Medicine, Hokkaido University School of Medicine, Kita 15 Nishi 7, Kitaku, Sapporo 060-8538, Japan*
[c]*Department of Human Genetics, Nagasaki University School of Medicine, Sakamoto 1-12-4, Nagasaki 852-8523, Japan*
[d]*Department of Orthopaedics, Nagasaki University School of Medicine, Sakamoto 1-12-4, Nagasaki 852-8523, Japan*

Received 12 December 1998

Abstract Peroxiredoxin (PRx) exhibits thioredoxin-dependent peroxidase activity and constitutes a family of proteins. Four members of genes from rat tissues were isolated by PCR using degenerated primers based on the sequences which encode a pair of highly conserved Cys-containing domains, and were then cloned to full-length cDNAs. These included two genes which have previously been isolated in rats, PRx I and PRx II, and two rat homologues of PRx III and PRx IV. We showed, for the first time, the simultaneous expression of all four genes in various rat tissues by Northern blotting. Since a discrepancy exists regarding cellular distribution, we further characterized PRx IV by expressing it in COS-1 cells. This clearly demonstrates that PRx IV is a secretory form and functions within the extracellular space.

Key words: Thioredoxin peroxidase; Secretory form; Reactive oxygen species

1. Introduction

Oxidative stress is caused as the result of a variety of physiological and pathophysiological conditions. Oxidative modifications of biological molecules such as proteins, lipids, and nucleic acids are potentially harmful to living organisms and may contribute to aging [1]. Since thiol groups are highly sensitive to oxidation, proteins in which thiol groups play a key role represent an important target of oxidative stress. Certain antioxidative enzymes such as the superoxide dismutases (SODs) and glutathione peroxidases (GPxs), and antioxidant molecules such as glutathione and thioredoxin serve to protect essential molecules against oxidative modification.

Recently, a novel family of proteins which function as antioxidants, in the protection of thiol groups in proteins, has been reported [2,3]. Since they also exhibit peroxidase activity in a thioredoxin (TRx)-dependent manner, they are referred to as peroxiredoxins (PRxs), and constitute a system which is similar to the glutathione/GPx system [4]. Members of this PRx family have two highly conserved Cys-containing domains, and are distributed widely in nature from yeast cells to mammalian tissues. Various biological effects have been reported with respect to these gene products in different species. Three types of PRxs are known in mammals. PRx I has heme binding capacity, and thus is called HBP [5,6]. This protein also acts as a natural killer cell-enhancing factor (NKEF-A) [7]. The second member of the PRx family, PRx II, is also able to act as a natural killer cell-enhancing factor (NKEF-B), and is often referred to as a thiol-specific antioxidant (TSA) [8–10]. These two members of the gene family are located in the cytosol. PRx III, which is found in mitochondria, was originally reported to be an indispensable factor involved in the differentiation of mouse MEL cells (MER5, now renamed AOP-1) [11]. This protein is also referred to as SP-22 because it is a substrate protein for a mitochondrial ATP-dependent protease [12]. The fourth member of the family, PRx IV (AOE372) [13], has been cloned only quite recently from human cells.

The issue of how many genes, and which member of the genes, are expressed in a single mammalian species is currently not clear. In this communication, we report on the PCR amplification of the PRx gene superfamily, using degenerated primers which correspond to two highly conserved Cys-containing domains, and the cloning of four cDNA members. Since there is still considerable debate on the characteristics of PRx IV, we further characterized this protein via its expression in COS-1 cells.

2. Materials and methods

2.1. Cell culture

COS-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL), 100 U/ml penicillin, and 100 U/ml streptomycin in a humid atmosphere of 5% CO_2 at 37°C.

2.2. PCR using degenerated primers

Total cellular RNAs were prepared from rat tissues as described previously [14]. Oligodeoxyribonucleotide primers were designed based on the nucleotide sequences which encode the highly conserved Cys-containing domains. These are Px1, 5′-ACNTTTGTGTGYCCYACNGA-3′ and Px2, 5′-GCNGGRCANACTTCNCCATG-3′ (N = G, A, T, or C; Y = C or T; R = A or G). Single strand cDNAs were synthesized from 10 μg total RNA and used as templates for PCR at 94°C for 30 s, 55°C for 60 s, and 72°C for 90 s for 25 cycles. This amplified an about 460 bp DNA fragment. These were subcloned into a pT7 Blue-T vector (Novagen) and sequenced using a computer-assisted automated sequencer (DSQ-1000L, Shimadzu). A homology search was executed on the BLAST/FASTA program provided via Internet by the National Library of Medicine, NIH (USA).

2.3. Isolation of cDNAs and genomic clones

About 10^6 independent plaques from a rat kidney cDNA library constructed in λgt10 [15] were screened with PRx probes labelled with

*Corresponding author. Fax: (81) (6) 879-3429.
E-mail: proftani@biochem.med.osaka-u.ac.jp

Abbreviations: ER, endoplasmic reticulum; GPx, glutathione peroxidase; NO, nitric oxide; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PRx, peroxiredoxin; SOD, superoxide dismutase; TRx, thioredoxin

PII: S0014-5793(98)01736-0

a random priming kit (Amersham) and [α-^{32}P]dCTP (Amersham). The 5′ end of the rat PRx IV was cloned by the rapid amplification of cDNA ends (RACE) method using a Marathon cDNA amplification kit (Clontech), since it was not possible to isolate a λ phage clone containing the 5′ region from the cDNA library. 10 μg of total RNA, isolated from rat liver, was reverse-transcribed with RAV-2 (Takara Co.) using the Px2 primer. After ligation of the 5′ adapter to the end of the reverse transcribed product, PCR was performed by using the 5′ adapter primer (5′-CCATCCTAATACGACTCACTATAGGGC-3′) and the gene-specific primer (5′-CCAAGTATTTCCCACGATAGTCGGT-3′). The 409 bp PCR products were subcloned into pT7 Blue-T vector and the nucleotide sequences determined for five independent clones. All were found to be identical. This cDNA fragment was ligated to the 5′ end of the partial PRx IV cDNA to construct full length cDNA. To isolate PRx IV genomic clones, 10^6 plaques of a λDASH II human genomic library (Stratagene) were screened with the rat cDNA fragment under high stringency conditions.

2.4. Fluorescence in situ hybridization (FISH)

FISH was performed on human R-banded metaphase chromosomes as described previously [16]. The isolated λ phage clone was labelled with biotin-16-dUTP using a nick-translation kit (Boehringer-Mannheim, Germany). Hybridized signals were observed under a fluorescence microscope with filters B2-A and B2-E (Nikon, Japan).

2.5. Northern blotting

Twenty microgram of total RNA was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde. The size-fractionated RNAs were transferred onto a Zeta-Probe membrane (Bio-Rad) by capillary action. After hybridization with the ^{32}P-labelled PRx probes at 42°C in the presence of 50% formamide, the membrane was washed at 55°C in 2×SSC (1×SSC: 150 mM NaCl and 15 mM sodium citrate, pH 7.5) containing 0.1% sodium dodecyl sulfate (SDS) for 80 min. The Kodak XAR films were exposed for 2 days with an intensifying screen at −80°C.

2.6. Preparation of anti-PRx IV antibodies

A portion of PRx IV cDNA encoding 175 amino acids, which encompasses the entire carboxy-terminal region, was ligated to a plasmid pMAL-c (New England BioLabs) and the fusion protein with maltose binding protein (MBP-PRx IV) was expressed in *Escherichia coli*. MBP-PRx IV was purified using an amylose resin affinity column (New England BioLabs) according to the manufacturer's instructions. The purified MBP-PRx IV was digested with Factor Xa (New England BioLabs) and separated from MBP by an AcA54 gel filtration

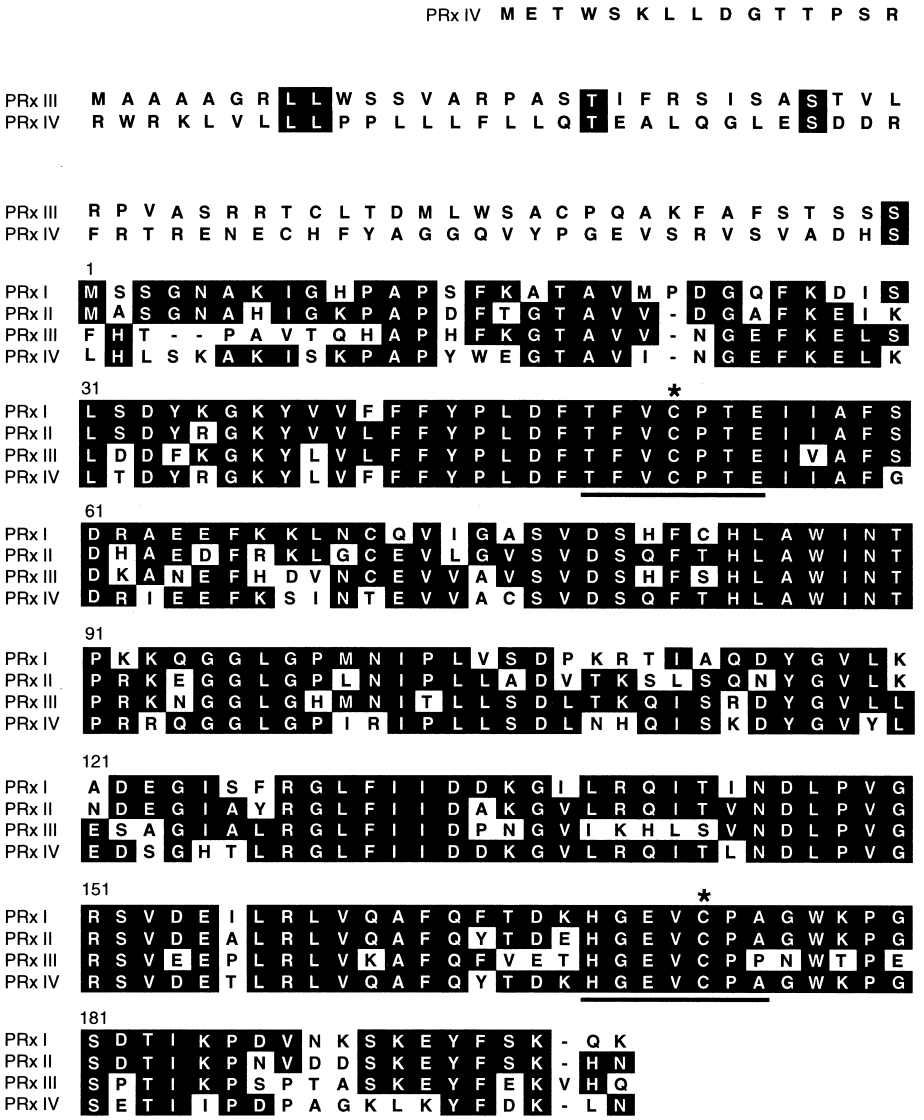

Fig. 1. Alignment of amino acid sequences among rat PRxs. The amino acid sequences applied for synthesizing degenerated oligonucleotide primers are underlined. Conserved, essential Cys residues are marked with asterisks. White letters in black boxes indicate the conserved residues among the family members. Nucleotide sequences for rat PRx III and PRx IV were deposited to GenBank with accession numbers AF106944 and AF106945, respectively.

column. Female rabbits were immunized subcutaneously with 200 μg PRx IV protein in Freund's complete adjuvant. The rabbit was bled 2 weeks after the last immunization. The IgG fraction was purified from the anti-sera by fractionation with ammonium sulfate. This anti-PRx IV IgG specifically recognized PRx IV.

2.7. Expression of PRx IV in COS-1 cells and immunofluorescent staining

Full length PRx IV cDNA was ligated into the pSVK3 expression vector carrying an SV40 early promoter (Amersham/Pharmacia Biotech). 50 μg of CsCl-purified pSVK3-PRx IV cDNA was transfected into COS-1 cells by the electroporation method using Gene Pulser (Bio-Rad) under conditions of 220 V and 960 μF in ice cold HEPES-buffered saline, pH 6.95. Six hours after transfection, the culture medium was replaced with fresh medium. At 48 h after transfection, culture media and transfected cells were harvested and used for immunoblot analyses. The chemiluminescence method was employed to amplify the signal using an ECL kit (Amersham).

For immunofluorescent staining, PRx IV cDNA-transfected COS-1 cells were seeded on a glass coverslip and cultured for 48 h. They were washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 30 min at 27°C, washed 3 times, and then incubated overnight with 1:500 diluted anti-rat PRx IV antibody in the permeabilization solution (0.2% Triton X-100, 3% BSA, and 0.05% sodium azide in 10 ml PBS) at 4°C. The samples were then washed 3 times with PBS, and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG at room temperature for 2 h. After washing 3 times with PBS, the cells on the coverslip were mounted on a slide glass and examined by fluorescence microscopy (AX70, Olympus).

3. Results

3.1. PCR amplification of a gene family using degenerated primers and cloning full length PRx cDNAs

A homology search on the reported amino acid sequences of members of PRx gene superfamily showed several highly homologous regions, especially two domains with essential Cys residues [4]. We designed degenerated primers Px1 and Px2, based on the nucleotide sequences which encode a pair of highly conserved Cys-containing domains, and amplified about 460 bp cDNAs which had been reverse transcribed from total RNAs extracted from rat brain and liver using these primers. As a result, we obtained cDNA fragments covering four members of the clones in the PRx gene superfamily.

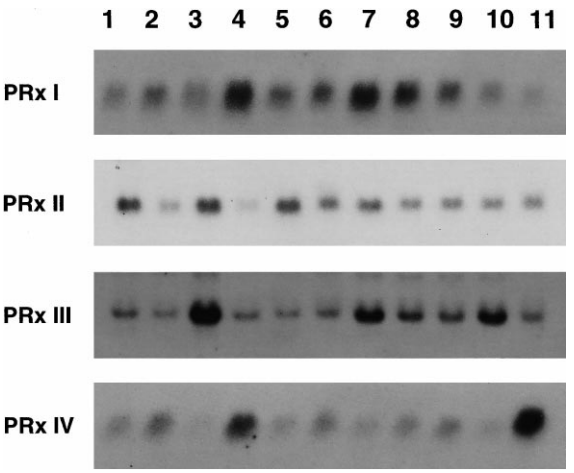

Fig. 2. Differential expression of PRx mRNAs in various tissues in rats. Total RNA (20 μg), isolated from 11 tissues of Sprague-Dawley rats at 14 weeks of age, were analyzed by Northern blotting using specific probes for rat PRxs genes. Lane 1: brain, 2: lung, 3: heart, 4: liver, 5: spleen, 6: stomach, 7: kidney, 8: small intestine, 9: colon, 10: skeletal muscle, 11: testis. The quality of RNA as judged by 18S rRNA was about the same in all samples. Samples from five different rats were analyzed and typical data are shown.

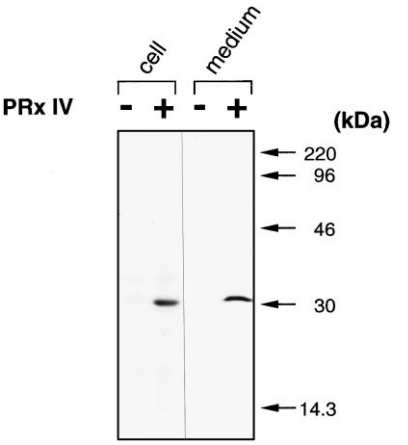

Fig. 3. Immunoblot analysis of PRx IV protein transiently expressed in COS-1 cells. pSVK3 expression vector carrying entire rat PRx IV cDNA was transfected into COS-1 cells by electroporation. 20 μg of proteins from a cell lysate or 10 μl of culture medium were separated on SDS-PAGE and then transferred onto nitrocellulose membrane. Anti-PRx IV IgG and peroxidase-conjugated anti-rabbit IgG were used as primary and secondary antibodies, respectively. The chemiluminescence method was employed to amplify the signal using an ECL kit.

A rat kidney cDNA library [15] was screened with these DNA fragments as probes and full length clones for three genes were obtained, corresponding to rat homologues of PRx I, PRx II, and PRx III. PRx I and II have previously been reported in rats, and PRx III represents a rat homologue reported in mouse. Since the fourth clone lacked the 5′ region, 5′-RACE was carried out to isolate the full length clone. The resultant clone was novel when cloned by us, but its human homologue was recently reported and referred to as AOE372 [13].

We then screened about 10^6 λ phages from a human genomic DNA library constructed in λDASH II using a rat PRx IV cDNA fragment as a probe and isolated three independent clones. Since the gene loci of three members of PRx except for PRx IV are already known [11,17,18], one of the genomic clones was used as a probe for FISH analysis on human R-banded metaphase chromosomes. This identified the human PRx IV unambiguously on chromosome Xp21.3 (data not

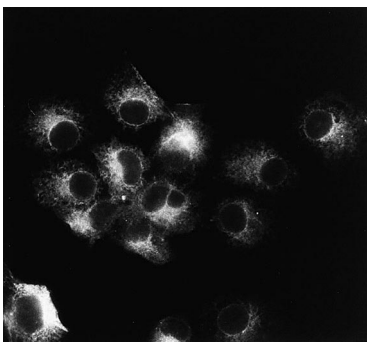

Fig. 4. Immunofluorescent staining of PRx IV expressed in COS-1 cells. After transfection of COS-1 cells with rat PRx IV cDNA, they were reacted with anti-PRx IV IgG. FITC-labelled anti-rabbit IgG was used as the secondary antibody. The cells were examined by fluorescent microscopy (×360).

shown), which is consistent with a recent report on this subject [19].

3.2. Characteristics of PRx proteins

Deduced amino acid sequences of these genes showed considerable similarity throughout the molecules, especially the two domains which contain essential Cys residues (Fig. 1). Hydropathy profiles of these gene products also confirmed the resemblance of their sizes and hydrophobicities except for the amino-termini of PRx III and PRx IV. Since mouse PRx III has been reported to be a mitochondrial protein, the extended amino-terminal sequences could act as a leader peptide for mitochondrial import. On the other hand, the amino-terminal hydrophobic stretch in PRx IV was typical for the signal sequence of secretory proteins or the transmembrane α-helix of type II membrane proteins.

3.3. Differential expression of PRx mRNAs in rat tissues

Although numerous functions have been reported for the PRx proteins, their actual physiological roles in each organ are still in debate. To understand the physiological role of PRx isozymes in individual tissues, we compared their multiplex expression in 11 tissues by using specific probes for all four isozymes and Northern blotting (Fig. 2). Samples from five different rats were analyzed and typical data are shown. PRx I, which was originally found to be a major heme binding protein in liver, showed higher levels of expression in liver, kidney, and small intestine than other tissues. Expression of PRx II was higher in heart, brain, and spleen, but lower in liver. PRx III was expressed at high levels in heart, skeletal muscle, and kidney, which are all rich in mitochondria. PRx IV was highly expressed in liver and testis. Thus all four isoforms appear to be expressed rather ubiquitously, although enhanced expression was observed for a particular isoform in some tissues.

3.4. Characteristics of PRx IV expressed in COS-1 cells

Since PRx IV contains the amino-terminal hydrophobic stretch, the possibility exists that post-translational processing could occur. To investigate the property of PRx IV, we attempted an immunoblot analysis of various cell lines, but failed to detect reliable signals, probably due to the low level of expression of PRx IV in these cells (data not shown). We then performed the transient expression of PRx IV in COS-1 cells. We carried out immunoblot analyses of cell lysates and conditioned medium using a specific anti-PRx IV antibody (Fig. 3). While untransfected COS-1 cells showed no detectable PRx IV protein in either the cell lysate or the conditioned medium, PRx IV cDNA-transfected cells clearly displayed specific bands corresponding to both of these. The size of the resultant PRx IV protein was 30 kDa, which corresponds to that of PRx IV truncated at the C-terminus of the amino-terminal hydrophobic stretch.

To determine the localization of the expressed PRx IV in cells, we then performed immunofluorescent staining. When COS-1 cells transfected with PRx IV cDNA were examined, an intracellular network-like structure, which is typical for endoplasmic reticulum (ER), was observed (Fig. 4). No staining of the plasma membrane or nuclei was observed. Untransfected COS-1 cells, used as controls, showed no signals. These data are completely consistent with the nature of PRx IV as a secretory protein.

4. Discussion

PRx has been characterized as a Trx-dependent peroxidase [4]. The PRx family have been cloned in various organisms from prokaryotes to mammalians [2,10]. Two domains, which contain essential Cys residues, responsible for their antioxidant activities were highly conserved among different species. Although a variety of functions has been reported for these gene products, the issue of how many genes exist in a single mammalian species, and which members of the genes are expressed in individual tissues remains unclear. In this communication, we report the cloning of two additional rat homologues and show that at least four PRxs are simultaneously expressed in rat tissues. While PRx I and II are cytoplasmic proteins [5,8], PRx III is localized in mitochondria [12]. The expression of PRx III was abundant in tissues that are rich in mitochondria, such as heart, kidney, and skeletal muscle, and whose oxygen consumption is relatively high. Since mitochondria are the main intracellular organelles which consume molecular oxygen by respiration, PRx III may function in concert with Mn-SOD to scavenge reactive oxygen species (ROS) which leak from the electron transport chain.

This study focused on PRx IV, whose human homologue was reported quite recently by two groups. Jin et al. [13] identified the gene product within cells, with a similar localization to PRx I, and concluded that it was a cytoplasmic protein. However, its hydropathy profile clearly showed a hydrophobic stretch at the N-terminus, which is typically found in secretory or type II transmembrane proteins. Haridas et al. [19] actually identified PRx IV in conditioned medium of Jurkat and HL60 cells. When we transfected COS-1 cells with PRx IV cDNA, immunoreactive bands to anti-PRx IV IgG were detected in both the conditioned medium and a cellular extract. Its size, 30 kDa, was smaller than the predicted size from the cDNA sequences but corresponded to the truncated polypeptide after the hydrophobic stretch. Thus, the recombinant PRx IV is probably processed to a secretory form post-translationally. Immunofluorescent staining of the cDNA-transfected cells gave a staining pattern which was typical for an ER structure (Fig. 4). Since the molecular size of the intracellular PRx IV was the same as that of the secreted one (Fig. 3), it is also predicted to exist as a truncated and soluble form in the ER, but not as a membrane-bound form. It is, therefore, conceivable that the protein which is localized in the ER is in the process of secretion as is commonly observed for other secretory proteins. Accordingly, we conclude that PRx IV represents the secretory form of the PRx family.

Among the functions reported for other members of the PRx gene products, only TRx-dependent peroxidase activity is commonly accepted as the enzymatic activity [20]. The same activity has also been reported for the human PRx IV homologue [13]. It is well established that significant amounts of TRx and TRx reductase are present in the extracellular space [21]. Hence, PRx IV would be able to function as a TRx-dependent peroxidase within the extracellular space in a manner similar to other antioxidant enzymes, such as plasma GPx and extracellular SOD. Since it is well known that the liver produces many plasma proteins such as albumin and transferrin, and expresses high levels of PRx IV mRNA, PRx IV may be secreted into plasma from the liver, thus protecting the vascular system from ROS.

Although Haridas et al. [19] reported inflammatory cytokine-like activities, such as the activation of NF-κB and the induction of nitric oxide synthase (NOS II), for PRx IV, an opposite effect was reported by Jin et al. [13] who showed suppression of NF-κB DNA binding activity in AOE372-overexpressing cells. In our preliminary experiment, recombinant rat PRx IV produced in a baculovirus/insect cell system did not induce NO production in RAW264.7 cells (Okado et al., submitted). Thus, a discrepancy remains as to its function. Further experiments will be required to solve these problems, and to comprehend the actual role of PRx IV in vivo.

Acknowledgements: We thank Prof. Akira Kitabatake, Cardiovascular Medicine, Hokkaido University School of Medicine, for beneficial advice. We also thank Drs. John M.C. Gutteridge and Milton S. Feather for editing the manuscript. This work was supported, in part, by a Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Science, Sports and Culture, Japan, and Research Grant (9A-1) for Nervous and Mental Disorders from the Ministry of Health and Welfare, Japan.

References

[1] Stadtman, E.R. (1992) Science 257, 1220–1224.
[2] Chae, H.Z. and Rhee, S.G. (1994) Biofactors 4, 177–180.
[3] Chae, H.Z., Robison, K., Poole, L.B., Church, G., Storz, G. and Rhee, S.G. (1994) Proc. Natl. Acad. Sci. USA 91, 7017–7021.
[4] Chae, H.Z., Chung, S.J. and Rhee, S.G. (1994) J. Biol. Chem. 269, 27670–27678.
[5] Iwahara, S., Satoh, H., Song, D.X., Webb, J., Burlingame, A.L., Nagae, Y. and Muller, E.U. (1995) Biochemistry 34, 13398–13406.
[6] Ishii, T., Yamada, M., Sato, H., Matsue, M., Taketani, S., Nakayama, K., Sugita, Y. and Bannai, S. (1993) J. Biol. Chem. 268, 18633–18636.
[7] Shau, H., Butterfield, L.H., Chiu, R. and Kim, A. (1994) Immunogenetics 40, 129–134.
[8] Chae, H.Z., Kim, I.H., Kim, K. and Rhee, S.G. (1993) J. Biol. Chem. 268, 16815–16821.
[9] Kim, K., Kim, I.H., Lee, K.Y., Rhee, S.G. and Stadtman, E.R. (1988) J. Biol. Chem. 263, 4704–4711.
[10] Lim, Y.S., Cha, M.K., Kim, H.K. and Kim, I.H. (1994) Gene 140, 279–284.
[11] Tsuji, K., Copeland, N.G., Jenkins, N.A. and Obinata, M. (1995) Biochem. J. 307, 377–381.
[12] Watabe, S., Kohno, H., Kouyama, H., Hiroi, T., Yago, N. and Nakazawa, T. (1994) J. Biochem. Tokyo 115, 648–654.
[13] Jin, D.Y., Chae, H.Z., Rhee, S.G. and Jeang, K.T. (1997) J. Biol. Chem. 272, 30952–30961.
[14] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[15] Nishikawa, A., Ihara, Y., Hatakeyama, M., Kangawa, K. and Taniguchi, N. (1992) J. Biol. Chem. 267, 18199–18204.
[16] Ohta, T., Tohma, T., Soejima, H., Fukushima, Y., Nagai, T., Yoshiura, K., Jinno, Y. and Niikawa, N. (1993) Hum. Genet. 92, 1–5.
[17] Prosperi, M.T., Apiou, F., Dutrillaux, B. and Goubin, G. (1994) Genomics 19, 236–241.
[18] Pahl, P., Berger, R., Hart, I., Chae, H.Z., Rhee, S.G. and Patterson, D. (1995) Genomics 26, 602–606.
[19] Haridas, V., Ni, J., Meager, A., Su, J., Yu, G.L., Zhai, Y., Kyaw, H., Akama, K.T., Hu, J., Van, E.L. and Aggarwal, B.B. (1998) J. Immunol. 161, 1–6.
[20] Kang, S.W., Chae, H.Z., Seo, M.S., Kim, K., Baines, I.C. and Rhee, S.G. (1998) J. Biol. Chem. 273, 6297–6302.
[21] Nakamura, H., Nakamura, K. and Yodoi, J. (1997) Annu. Rev. Immunol. 15, 351–369.